Peer Review File

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Reviewer A

Introduction

Comment 1. Line 33. Authors should briefly include some current immunotherapies available for treating gliomas, such as those outlined in Gallus et al (2023). Gallus M, Kwok D, Lakshmanachetty S, Yamamichi A, Okada H. Immunotherapy Approaches in Isocitrate-Dehydrogenase-Mutant Low-Grade Glioma. Cancers (Basel). 2023 Jul 22;15(14):3726. doi: 10.3390/cancers15143726. Erratum in: Cancers (Basel). 2023 Dec 26;16(1): PMID: 37509387; PMCID: PMC10378701.

Reply 1: Dear Professor, thank you very much for your constructive comments. The paper is related to immunotherapy, and indeed, the introduction should be supplemented with background on glioma immunotherapy. Following your suggestions, we have made the necessary modifications. If our explanations have not met your expectations, please be sure to notify us. We will continue to revise the paper and provide further explanations.

Change in the text: We added the sentence “Therefore, there is an urgent need for new therapeutic approaches. In recent years, immunotherapeutic strategies have achieved significant success across various types of cancer. However, the efficacy of immunotherapy including anti-PDL-1 immune checkpoint blockade, vaccine immunotherapy against glioma-associated antigens in treating gliomas has been constrained by several challenges, including tumor heterogeneity and the immunologically 'cold' tumor microenvironment. Therefore, regulating the LGG tumor microenvironment to enhance its sensitivity to immunotherapy is a critical issue that urgently needs to be addressed.” in the first paragraph of the introduction section. The reference has been cited.

Comment 2. Several studies have shown disappointing responses to TGF-β-based therapies in cancer, including gliomas. This is an important issue that needs to be addressed. This present in-silico analysis may provide a rationale for better understanding of LGG subtypes and provide more targeted approaches. Please refer to these publications:

Teixeira AF, Ten Dijke P, Zhu HJ. On-Target Anti-TGF-β Therapies Are Not Succeeding in Clinical Cancer Treatments: What Are Remaining Challenges? Front Cell Dev Biol. 2020 Jul 8;8:605. doi: 10.3389/fcell.2020.00605. PMID: 32733895;

PMCID: PMC7360684.
Cheng-Yi Huang, Chih-Ling Chung, Tsung-Hui Hu, Jih-Jung Chen, Pei-Feng Liu, Chun-Lin Chen, Recent progress in TGF-β inhibitors for cancer therapy, Biomedicine &Pharmacotherapy, Volume 134, 2021, 111046, ISSN 0753-3322, https://doi.org/10.1016/j.biopha.2020.111046.
Metropulos AE, Munshi HG, Principe DR. The difficulty in translating the preclinical success of combined TGFβ and immune checkpoint inhibition to clinical trial. EBioMedicine. 2022 Dec;86:104380. doi: 10.1016/j.ebiom.2022.104380. Epub 2022 Nov 28. PMID: 36455409; PMCID: PMC9706619.
Reply 2: Thanks for your advice. The mentioned references have been added. Furthermore, we also presented the current status of TGF-βbased treatment in gliomas and objectives for TGF-βsubtype for LGG.
Change in the text: In the second paragraph, we added that “Furthermore, several studies have demonstrated lackluster responses to TGF-β-based therapies in various cancers, including gliomas, underscoring a critical issue that demands attention. The current in-silico analysis might offer a foundation for improving our understanding of LGG subtypes and developing more focused therapeutic strategies”.
Methods.

Comment 3. Lines 252-258. Please provide sufficient details for quantifying RNA-seq determinations and methods of normalization (e.g., RSEM, TPM).
Reply 3: Dear professor, thanks for your suggestion. We have provided sufficient details for quantifying RNA-seq determinations and methods of normalization.
Change in the text: In the first and second paragraph of the section of methods, we added that “RNA-seq is a powerful method for transcriptomes quantitatively. Here are the key aspects to consider when quantifying RNA-seq data and the common methods of normalization. Library Preparation: RNA samples are converted into a library of cDNA fragments with adapters attached to each end. The choice of library preparation method (e.g., poly-A selection, ribosomal RNA depletion) can affect which RNA species are sequenced. Sequencing: This step involves sequencing the cDNA fragments to generate raw sequence reads. Sequencing depth (i.e., the number of reads) is crucial for quantification, as it impacts the ability to detect and accurately quantify low-abundance transcripts. Mapping Reads: The raw reads are aligned to a reference genome or transcriptome. The efficiency of this step depends on the quality of the reads and the reference sequence used. Counting: Once reads are mapped, counts are generated for each gene or transcript, representing the number of reads or fragments aligned.

There were several Methods of Normalization as follow. TPM (Transcripts Per Million): Normalizes for total library size and gene length, making it useful for

comparing transcript levels within samples. RPKM/FPKM (Reads/Fragments Per Kilobase of transcript per Million mapped reads): Similar to TPM but less suitable for comparing across samples because it doesn't account for differences in sequencing depth and library composition between samples. TMM (Trimmed Mean of M-values): Used in edgeR, it normalizes based on the assumption that most genes are not differentially expressed. It adjusts for differences in library composition. Quantile Normalization: This method assumes the same distribution of gene expression across samples and adjusts the data accordingly. DESeq's Size Factor Normalization: Used in DESeq, this method estimates size factors based on the median ratio of gene counts relative to a geometric mean per gene (thus stabilizing variance across samples). The data used in this article were extracted from public databases, so quantification of RNA seq is not involved. This study used packages such as DESeq2 and Limma to normalize expression levels between different samples, eliminating the impact of experimental conditions and instrument differences on the data, and correcting systematic errors between different experimental batches."

Comment 4. Lines 267-271. Please provide more details for the methods and parameters used for the R packages.

Reply 4: Dear Professor, thank you very much for your constructive comments. The "ConsensusClusterPlus" R package consists of numerous lines of code, which would be too verbose to display in full within the methodology section. Therefore, we have opted to only provide a description of the workflow of this package and the references in the methods section.

Change in the text: In the section of **Construction and validation of TGF-β subtypes in the methods, we added that "**ConsensusClusterPlus processes a numerical data matrix, organizing items in columns and features in rows. This function selectively subsamples the matrix based on pItem, pFeature, weightsItem, and weightsFeature, and partitions the data into clusters ranging from 2 to maxK using the specified clustering algorithm in clusterArg. It supports both agglomerative hierarchical clustering (hclust) and k-means clustering, with additional configurations available as described above".

Comment 5. Line 277. Please elaborate on the statement "It is worth noting that transcriptome data must be normalized".

Reply 5: Dear Professor, in this study, we utilized TIME2.0 to calculate the immune cell infiltration for all samples. Prior to the calculation of immune cell infiltration, the website requires the upload of a gene expression matrix that has not been log-transformed and has been standardized using TPM normalization.

Change in the text: No changes.

Comment 6. Please provide brief details of how "GSVA" was used to calculate the metrics.

Reply 6: Thanks, we have provided the details for “GSVA” operational principle.

Change in the text: In the section of **Estimation of immune cell infiltration in methods, we added that “**GSVA is a non-parametric, unsupervised method used for estimating variations in gene set activity across samples in genomic datasets. When calculating metrics with GSVA, a collection of gene sets is first defined. GSVA then assesses the variation in expression levels of these gene sets within individual samples to calculate an enrichment score for each gene set. This approach is applicable to RNA-seq and microarray data, enabling researchers to globally understand changes in biological processes or signaling pathways under various conditions. The outcomes of GSVA can be utilized for further differential expression analysis or as candidates for biomarkers**”.**

Comment 7. Line 292. How was the risk score used to identify High and Low-risk patients?

Reply 7: Dear Professor, in this article, we use the median value to divide all patients into high-risk and low-risk groups.

Change in the text: No changes.

Comment 8. Line 323. How were the assumptions of the Cox regression models tested?

Reply 8: In the context of Cox regression models, testing the assumptions typically focuses on checking the proportional hazards assumption. This is central to the model's validity and can be assessed through several methods: (1) **Graphical checks**: Plotting Schoenfeld residuals against time can indicate whether there's a trend, which suggests that the proportional hazards assumption may be violated. If the residuals are randomly scattered around zero without any apparent trend, it generally supports the assumption. (2) **Statistical tests**: The Schoenfeld residuals can also be used in a formal test. This involves correlating the residuals with time to see if the correlation is significantly different from zero. A significant correlation implies that the hazards are not proportional. (3) **Time-dependent covariates**: Incorporating interactions of covariates with a function of time into the model can check for non-proportionality. If these interaction terms are statistically significant, it suggests that the effect of the covariates is not constant over time, thus violating the proportional hazards assumption. (4) **Global tests**: These are tests designed to evaluate the overall fit of the Cox model and to check for deviations from the proportional hazards assumption across all covariates simultaneously. It is crucial for the reliability of the Cox model's results to verify that these assumptions hold or to adjust the model accordingly if they do not.

Change in the text: NA

Results.

Comment 9. Please provide the targets or mode of action for the drugs mentioned in lines 102 – 105.
Reply 9: Dear Professor, the "pRRophetic" R package predicts potential sensitivity to drugs based on differentially expressed genes between groups. However, it does not specify the potential targets for each drug. We hope that future algorithmic systems will be further refined to provide more detailed information.
Change in the text: NO changes.

Comment 10. Line 148. Typo for “Sub2 and Sub2”. Replace “Sub2” with “Sub3”. The results section does not clarify and detail how Sub1/2/3 were determined and leads to substantial confusion between TGFB cluster subtype and IDH status subtype. This issue needs to be explained throughout the manuscript.
Reply 10: Dear professor, the definition of the TCGA subtype was primarily based on status of IDH mutation and 1p19q codeletion. However, the definition of TGF-b cluster was obtained TGF-b associated genes. This information had been provided in the manuscript.
Change in the text: The “Sub2” has been replaced with “Sub3”. Furthermore, in the section of Construction and validation of TGF-β subtypes in methods, we added that “In the presented manuscript, we assessed the association between TGF-β the TCGA subtype. TCGA subtype 1(Sub1) was defined as IDH mutation and 1p19q codeletion, Sub2 was defined as IDH mutation and 1p19q non-codeletion, while IDH wildtype no matter what the status of 1p19q was defined Sub3.”

Discussion
Comment 11. Line 173. Please indicate which genes (BMP2, COL3A1, INHBB, MYC, NOG, PRKCZ, SERPINE1, SMAD9, TGIF2, THBS2, TNF, and TP53) exhibited positive and negative prognoses.
Reply 11: Dear reviewer, thanks for your construction. The prognostic information for those gene had provided in the manuscript.
Change in the text: In the second paragraph of the discussion section, we added that “Five of the 12 genes were positive factors associated with prognosis (BMP2, MYC, NOG, PRKCZ and SMAD9), while the remaining seven were risk factors (COL3A1, INHBB, SERPINE1, TGIF2, THBS2, TNF, and TP53).”

Comment 12. Lines 206 – 217. Two issues need to be addressed in this discussion: TGFB genes exert their effects through M1 to M2 Macrophage polarization. Do the

conclusions from the single-cell analysis point to a further understanding of this process from the most significantly affected pathways? Furthermore, are any of the prognostic and predictive markers identified in this study correlated with well-established targets of immune therapies (e.g., PD-L1, PD1, TIGIT etc.) that may inform the type of immune therapies that could be employed? The authors need to provide context to the immune therapies currently being employed in clinical trials.

Reply 12: Dear professor, thanks for your constructive advice. We provided the information for TGF-B associated genes affected M1 to M2 Macrophage polarization and signal pathway. Furthermore, the correlations between prognostic genes and targets of immune therapies (e.g., PD-L1, PD1, TIGIT etc.) were assessed. The results were provided in the first paragraph of results. Finally, we briefly summarized the immune therapies currently being employed in clinical trials in the second paragraph of the discussion section.

Change in the text: In the third paragraph end of discussion section, we added that "Furthermore, TGFB genes exert their effects through M1 to M2 Macrophage polarization. Based on our analysis of cellular communication, we found that TGF-related genes primarily regulate the tumor microenvironment, including M1 and M2 macrophages, through the JAM, APP, GRN, PSAP, and MIF signaling pathways. However, the specific mechanisms and the particular genes' actions on these pathways require further validation through cellular experiments. This may provide evidence for modulating the immune microenvironment in LGG." In the first paragraph end of results section, we added that "We then assess the correlation of those genes with immune checkpoint including PD1, PDL1 and TIGIT. Our results revealed that SERPINE1 is positively correlated with the expression of PDL1 and PD1, while NOG and BMP2 are negatively correlated with PDL1. The remaining correlations are not significant" and discussed that "In the presented study, we observed that SERPINE1, a TGF-β gene, is positively correlated with the expression of PDL1 and PD1, which suggested that SERPINE1 may be a potential target for enhancing immunotherapy. Future research should further evaluate how SERPINE1 regulates immunotherapy in gliomas" in the third paragraph in the discussion section. Furthermore, we presented that "Certainly, the heterogeneity of the tumor microenvironment in low-grade gliomas (LGGs) presents a challenge to current immunotherapy strategies. This variability may account for the wide range of treatment responses among patients and could help explain why the results of clinical trials have not met expectations. Current immunotherapies, such as inhibitors of the PD-1/PD-L1 and CTLA-4 pathways, tumor vaccines, immunomodulators, and cell therapies, have shown success in certain types of cancers, but their efficacy in LGGs has been limited. This may be partly due to the unique immunosuppressive microenvironment characteristic of LGGs. This environment is typically characterized by a low infiltration of immune cells, which may

also exhibit an immunosuppressive phenotype. To enhance therapeutic outcomes, one future research direction may involve a detailed characterization of the cellular components within the LGG tumor microenvironment, including immunosuppressive cells like regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs). Another research avenue is to define the molecular pathways within the tumor microenvironment, such as cytokines and chemokines that may regulate immune cell infiltration and function. With a better understanding of these cellular and molecular changes, we may identify new therapeutic targets that could be key in modulating the tumor microenvironment and improving response rates in LGG patients. For example, SERPINE1, as a regulator of immune responses and extracellular matrix remodeling, could be a potential target. By targeting SERPINE1 or its related pathways, we might be able to modulate the tumor microenvironment and enhance the efficacy of immunotherapies. More broadly speaking, efforts to improve treatment strategies for LGG will require the use of high-throughput tissue analysis techniques, integrating data from immuno-histochemistry, genomics, transcriptomics, proteomics, and single-cell analyses to build a more comprehensive model of the disease. Through such multidimensional analysis, we can gain a deeper understanding of the biological characteristics of LGG and the potential mechanisms underlying treatment failure, offering more personalized and effective treatment options for patients".

Figures.

Comment 13. Figure 2. Provide the sample size, median, and 95% CI for panels B and E in text.

Reply 13: Thanks, the information had been provided in the manuscript.

Change in the text: In the title of Figure 2, we corrected the previous title as "Performance of a consensus cluster to obtain three TGF-β subtypes. (a, d) the distribution of the 12 differential and prognostic TGF-β-related genes, clinical characteristics among the TGF-β subtypes in TCGA and CGGA datasets, respectively; (b, e) survival difference between TGF-β subtypes in TCGA(The median OS for A, B and C was 6.2, 2.1 and 8.2 years, respectively; HR(95%CI) for B vs. A: 0.58(0.37-0.91); HR(95%CI) for C vs. A: 3.25(2.07-5.10)) and CGGA(The median OS for A, B and C was 7.2, 1.9 and 9.8 years; HR(95%CI) for B vs. A: 0.65(0.48-0.86); HR(95%CI) for C vs. A:2.50(1.85-3.32)) datasets, respectively; (c, f) PCA."

Comment 14. Figure 6. How were sub1, sub2 and sub3 determined? Please resolve why the sample sizes vary between panels b, c and d.

Reply 14: Thank you. The information for sub1, sub2 and sub3 determination was provide in the section of **Construction and validation of TGF-β subtypes in methods.**

As for the sample sizes vary between panels b, c and d in Figure 6, there are some samples that lack certain clinical information in the TCGA database. As a result, there is a discrepancy in the number of samples when analyses are carried out with particular variables as the primary focus. For example, TCGA classifications are made on the basis of IDH mutation status and 1p/19q co-deletion status. However, a subset of samples does not have information regarding these two molecular markers, and thus these samples are excluded. This leads to inconsistencies in the clinical sample size.

Change in the text: In the section of **Construction and validation of TGF-β subtypes in methods., we added that "**In the presented manuscript, we assessed the association between TGF-β the TCGA subtype. TCGA subtype 1(Sub1) was defined as IDH mutation and 1p19q codeletion, Sub2 was defined as IDH mutation and 1p19q non-codeletion, while IDH wildtype no matter what the status of 1p19q was defined Sub3**".**

15. Figure 7. Provide the sample size, median, and 95% CI for panels. Change "correlationship" to correlations.

Reply 15: Thanks for your constructions. The sample size for low riskscore and high riskscore group were presented in the Figure 7a and 7b.

Change in the text: We added the median, and 95% CI(The prognostic and predictive role of risk score. (a and b) The prognostic effect of risk score in patients with LGG of TCGA (The median OS for patients high riskscore is 2 years, but 8 years for low riskscore, HR:1.494; 95%CI:1.349-1.654) and CGGA cohorts(The median OS for patients high riskscore is 3 years, but 10 years for low riskscore, HR:1.232; 95%CI:1.168-1.300), respectively) for Figure 7a and 7b in the Tittle for Figure 7.

16. Figure S10. Please provide the regression groups for the categorical variables and sample sizes for each group, and do they correspond to the sample sizes indicated in Tables S1 and S2?

Reply 16: Thanks. The sample size for age, grade, sex, IDH, 1P19Q codeletion, MGMT promoter methylation, radiation and chemotherapy were correspond to the sample sizes provided in the Table S1 and S2. Furthermore, we also provided sample size for low risk and high-risk groups. The Figure 7a and 7b showed sample size for low risk and high-risk groups in TCGA and CGGA cohorts, respectively.

Change in the text: No changes.

Reviewer B

1. Figures

a) Please check the citation.

211 TCGA dataset (Figure 32a-i). Principal components analysis (PCA) was employed to assess the resilient

b) Please check if here should be figure S8c and d.

270 and 1p19q no codeletion; sub3: IDH1 wildtype, Figure 8c and d). The survival analysis indicated that all

Reply: Thank you. The correct sequence of images has been adjusted.
Changes in the text: The Figure 32a-i has been corrected as “Figure S3a-i” and Figure 8c and d have been corrected as Figure S8c and d.

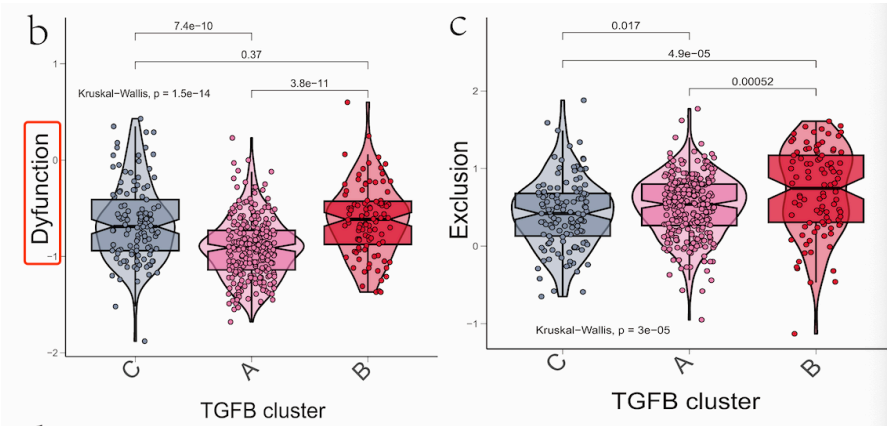
2. Figure 3

a) Please provide the meaning of the symbol “*, **, ***” in the legend.
b) Please define ns in the legend.
Reply: Thank you.
Changes in the text: The definition for symbol l “*, **, ***” and ns in the legend have been added in the Figure3 legend as following: *indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant.

3. Figure 4

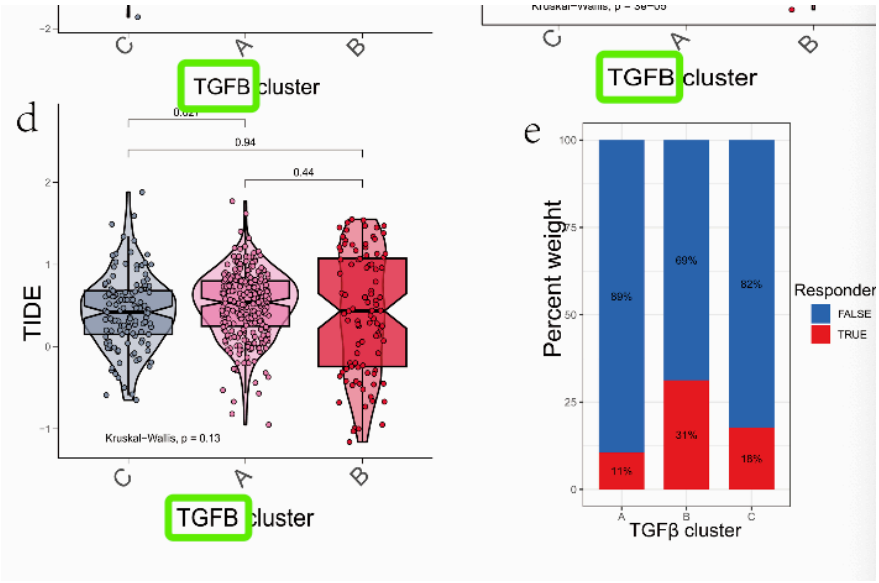
a) Please check the word “dysfunction”, it is different from the figure.
b) Please check the legend, it seems that there’s no IFNG in the figure.

between immune regulatory genes and TGF-β subtypes; (b-e) the dysfunction, IFNG, exclusion, and



c) In the figure, please check if it should be TGF-β.

605 TIDE score among TGF-β subtypes.



Reply: Dear editor, we performed correction based your suggestions.

Changes in the text: The dyfunction in Figure 4d has been corrected as “Dysfunction”, The IFNG has been deleted from the Figure 4 legend. As for the TGFB cluster, To maintain consistency and standardization throughout the text, we corrected TGFB or βcluster as TGF cluster. This name will be used consistently throughout the manuscript.

4 Figure 5

a) Please also provide the description for 5f in the legend.

b) Please provide the meaning of the symbol “***” in the legend.

c) Please define ns in the legend.

Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: The description for Figure 5f has been provided in the legend. At the end of Figure 5 legend, we added that “*indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant”.

5. Figure 6

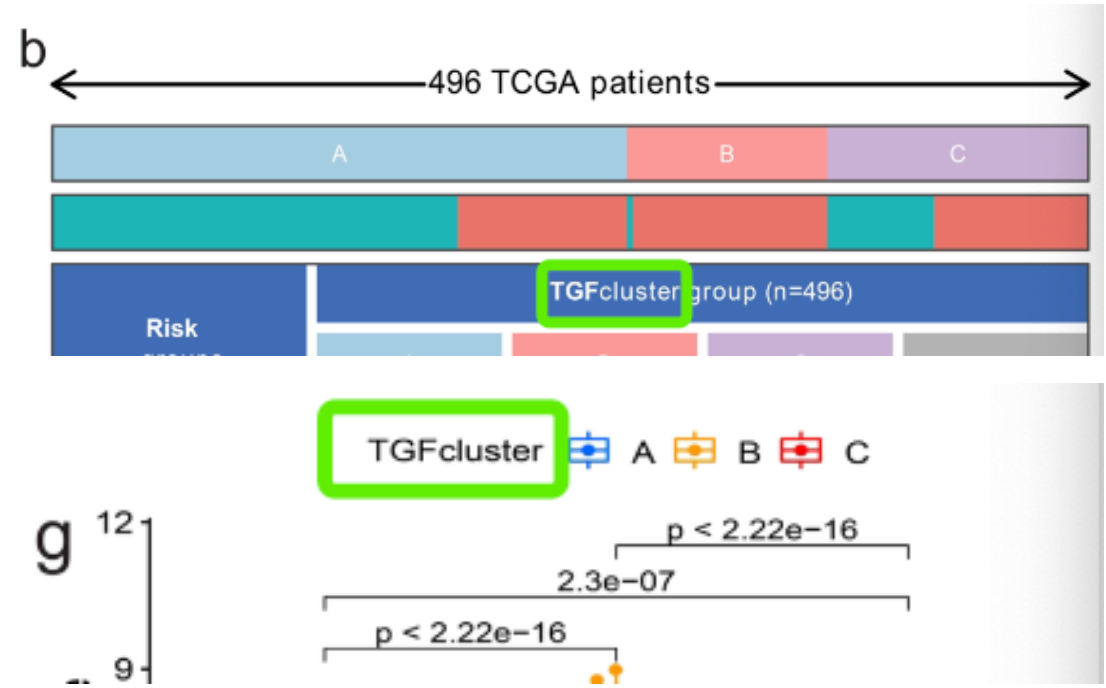
a) Please provide the meaning of the symbol “***” of 6A in the legend.

b) Please check if the figure matches the legend. TGF-β cluster or TGF cluster?

615 between low risk group and high risk group in TCGA dataset; (b, c and d) the relationship of TGF-β

616 cluster, tumor grade, and subtype associated with risk group; (e,f and g) the distribution of risk score

617 within TGF-β cluster, tumor grade, and subtype, respectively.

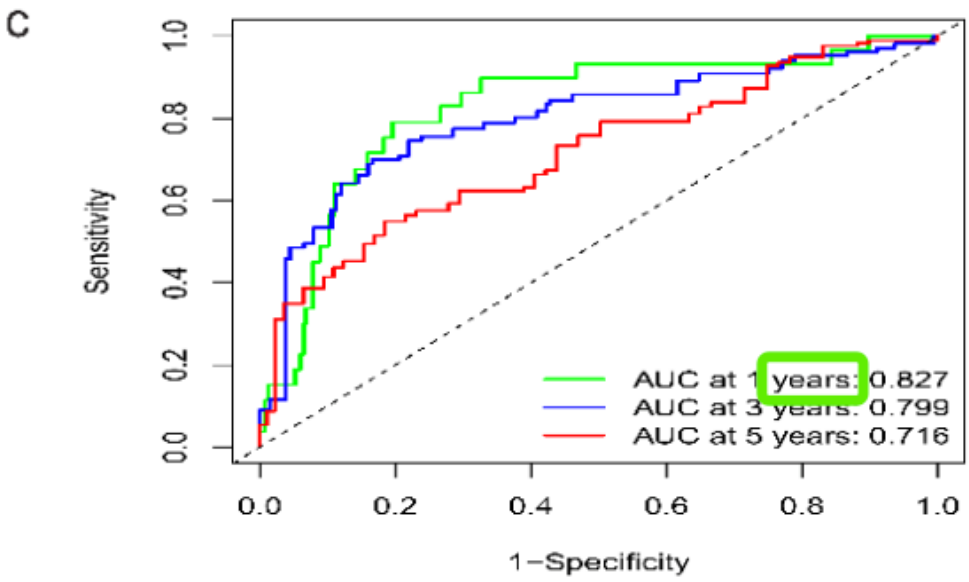


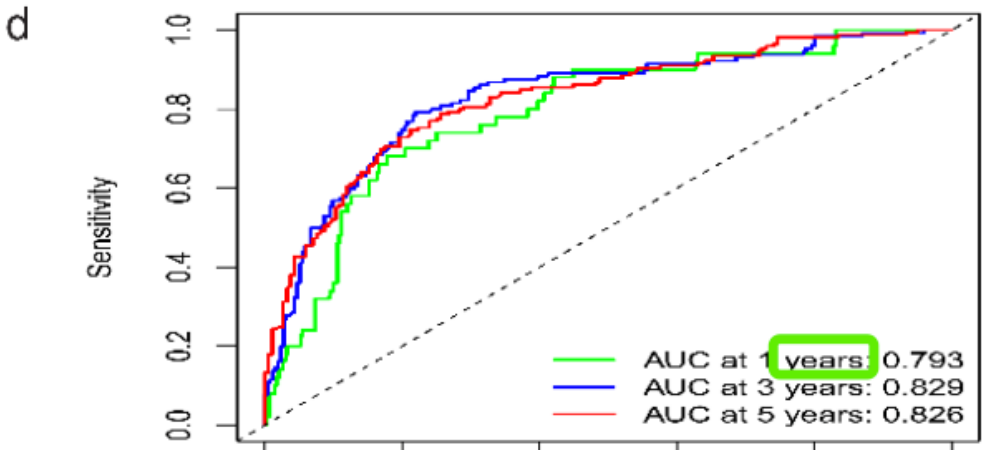
Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: At the end of Figure 6 legend, we added that "*indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant". As for the TGFB cluster, To maintain consistency and standardization throughout the text, we corrected TGFB or βcluster as TGF cluster. This name will be used consistently throughout the manuscript.

7. **Figure 7**

Please revise "1 years" to "1 year".





Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: The 1 years in Figure 7c and d have been corrected as 1 year. The latest version will be sent to you.

8. Figure S4

Please provide the meaning of the symbol "***" in the legend.

Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: At the end of Figure S4 legend, we added that "*indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant".

9. Figure S8

a) Please provide the meaning of the symbol "***" in the legend.

b) Please check if the figure matches the legend. TGF-β cluster or TGF cluster?

655 three genes in TGF-β cluster; (d) the expression of the three genes in subtypes based on IDH mutation

c) Please check if here should be (e, f, and g).

656 and 1p19q co-deletion; (e, j, and f) Survival graph describing the survival of SHD, SNCG, and

657 AC062021.1 in patients with LGG of TCGA cohort; (h,i, and j) Survival graph describing the survival

Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: At the end of Figure S8 legend, we added that "*indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant". As for the TGFB cluster, to maintain consistency and standardization throughout the text, we corrected TGFB or βcluster as TGF cluster. This name will be used consistently throughout the manuscript. Furthermore, (e,j and f) have been corrected as (e,f and g).

10. Figure S10

Please check if the figure matches the legend.

672 of each signature gene; (b) partial likelihood deviane of each signature gene; (c) Prognosis of SNCG,

673 SHA, and AC062021.1 in LGG patients by univariate analysis; (d) Heatmap showing the expression

Reply: Thank you. We have corrected it according to your opinion.

Changes in the text: We have corrected SHA as SHD.

11. Figure S12

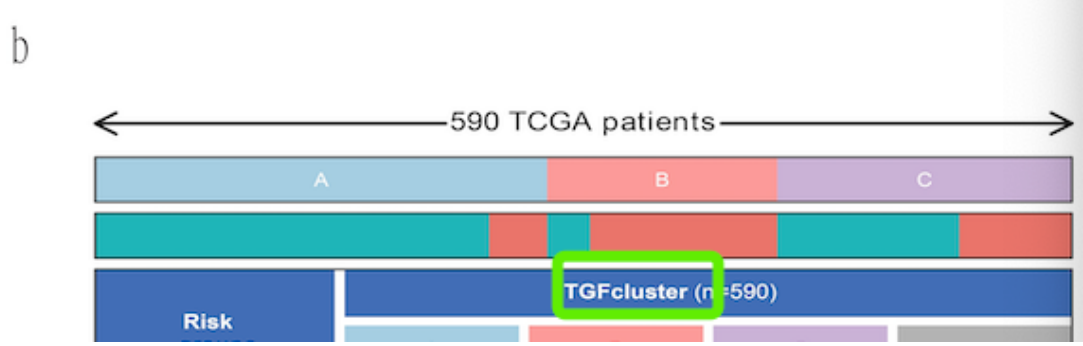
a) Please provide the meaning of the symbol "***" in the legend.

b) Please check if the figure matches the legend. TGF-β cluster or TGF cluster?

679 between low risk group and high risk group in TCGA dataset; (b, c and d) The relationship of TGF-β

680 cluster, tumor grade, and subtype associated with risk group; (e,f and g) the distribution of risk score

681 within TGF-β cluster, tumor grade, and subtype, respectively.



Reply: Thank you. We have corrected it according to your opinion.
Changes in the text: At the end of Figure S12 legend, we added that "*indicated p<0.05; **indicated p<0.01; ***indicated p<0.001; ns: Not significant". As for the TGFB cluster, to maintain consistency and standardization throughout the text, we corrected TGFB or βcluster as TGF cluster.

12. Main text and figures

a) As SHA and SHD are appeared in the both figures and main text, some are not matched. Please check the whole text and all figures.
b) Please also check TGF-β cluster and TGF cluster through the whole text and figures.
c) Please note that the subtitle "Methods and Materials" has been revised to "Method" as required by the journal.
Reply: Thank you. We have corrected it according to your opinion.
Changes in the text: We have corrected SHA as SHD in the manuscript. As for the TGFB cluster, to maintain consistency and standardization throughout the text, we corrected TGFB or βcluster as TGF cluster. The subtitle "Methods and Materials" has been revised to "Method" as required by the journal.

13. Table S1 and S2

Please add the description to the table footnote that how the data are presented in table. E.g., Data are presented as n(%).
Reply: Thank you. We have corrected it according to your opinion.
Changes in the text: At the end of the legends of Table S1 and S2, we added that Data are presented as n(%).